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Kiran Chintakayala
University of Nottingham

Cristina Machón
University of Nottingham

Anna Haroniti
University of Nottingham

Marilyn A. Larson
University of Nebraska Medical Center, malarson@unmc.edu

Steven H. Hinrichs
University of Nebraska Medical Center, shinrich@unmc.edu

See next page for additional authors

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Allosteric regulation of the primase (DnaG) activity by the clamp-loader (τ) in vitro

Kiran Chintakayala,[1] Cristina Machón,[1] Anna Haroniti,[1] Marilyn A. Larson,[2] Steven H. Hinrichs,[2] Mark A. Griep,[3] and Panos Soultanas[1]

[1] Centre for Biomolecular Sciences, School of Chemistry, University of Nottingham, Nottingham NG7 2RD, UK
[2] Department of Microbiology and Pathology, 986495 University of Nebraska Medical Center, Omaha, Nebraska 68198–6495, USA
[3] Department of Chemistry, University of Nebraska–Lincoln, Nebraska 68588–0304, USA.

Corresponding author — P. Soultanas; tel 44 0 115 9513525; fax 44 0 115 8468002; e-mail panos.soultanas@nottingham.ac.uk

Abstract
During DNA replication the helicase (DnaB) recruits the primase (DnaG) in the replisome to initiate the polymerization of new DNA strands. DnaB is attached to the τ subunit of the clamp-loader that loads the β clamp and interconnects the core polymerases on the leading and lagging strands. The τ–DnaB–DnaG ternary complex is at the heart of the replisome and its function is likely to be modulated by a complex network of allosteric interactions. Using a stable ternary complex comprising the primase and helicase from *Geobacillus stearothermophilus* and the τ subunit of the clamp-loader from *Bacillus subtilis* we show that changes in the DnaB–τ interaction can stimulate allosterically primer synthesis by DnaG in vitro. The A550V τ mutant stimulates the primase activity more efficiently than the native protein. Truncation of the last 18 C-terminal residues of τ elicits a DnaG-stimulatory effect in vitro that appears to be suppressed in the native τ protein. Thus changes in the τ–DnaB interaction allosterically affect primer synthesis. Although these C-terminal residues of τ are not involved directly in the interaction with DnaB, they may act as a functional gateway for regulation of primer synthesis by τ-interacting components of the replisome through the τ–DnaB–DnaG pathway.

Introduction

A multi-protein replisome complex carries out DNA replication in bacteria. The replisome is powered by a replicative helicase that uses the energy from ATP binding and hydrolysis to translocate forward separating the parental duplex in the process while a primase, attached to the replisome via the helicase, synthesizes short Okazaki fragments that initiate lagging strand synthesis (Johnson and O'Donnell, 2005). Complex interactions of replisomal components with each other and/or the DNA substrate co-ordinate the structural and functional competence of the replisome. The bacterial clamp-loader ($DnaX_3\delta\delta'\chi\psi$), the replicative helicase (DnaB) and the primase (DnaG) form the structural and functional core of the replisome. The helicase acts as the connector between the DnaX polypeptide of the clamp-loader and the primase in this ternary complex. A translational frameshift in the *Escherichia coli* (Flower and McHenry, 1990) and a transcriptional slippage in the *Thermus thermophilus* (Yurieva et al., 1997) *dnaX* genes, respectively, produce two variants of the DnaX protein; the full-length τ and shorter-version γ polypeptides. The latter lacks a distinct C-terminal domain (Cτ) that interacts with DnaB (Gao and McHenry, 2001a) and the α subunit (Kim and McHenry, 1996; Dallmann et al., 2000; Gao and McHenry, 2001b; Jergic et al., 2007; Xun-Cheng et al., 2007) of the core polymerase (αεθ). In *Streptococcus pyogenes* and other Gram-positive organisms the *dnaX* gene is assumed to code only for the full-length τ polypeptide (Bruck and O'Donnell, 2000), despite the fact that in the *Bacillus subtilis dnaX* gene a Shine–Dalgarno sequence is present upstream of a potential frameshift signal that could produce the shorter γ polypeptide (Haroniti et al., 2004). Indeed, *B. subtilis*τ has a domain organization similar to that of *E. coli*τ with a distinct Cτ that interacts with the *Geobacillus stearothermophilus* DnaB (Haroniti et al., 2004) and the bacteriophage SPP1 replicative DNA helicase G40P (Martínez-Jiménez et al., 2002). Although the two proteins share a high degree of identity in the first two-thirds of their sequences the homology is weaker in their Cτ sequences (Martínez-Jiménez et al., 2002). Overall the *E. coli* and *B. subtilis* τ proteins are 27% identical and 42% similar. By contrast, the *G. stearothermophilus* and *B. subtilis* proteins are 54% identical and 73% similar in their Cτ domains with their

homology rising to 78% identical and 86% similar in the rest of the τ sequence. The weak sequence conservation between the Cτ domains of the *E. coli* and the *Bacillus* proteins is not necessarily an indication of structural variability. For example, the apparently non-homologous N-terminal and C-terminal domains of DnaB and DnaG, respectively, have very similar structures (Soultanas, 2005). It may still be the case that the Cτ domains from *Bacillus* τ proteins have structures similar to that of the *E. coli* Cτ. Indeed, the NMR structure of domains IVa-V (equivalent to Cτ) of the *E. coli* τ revealed a KH type II fold with certain sequence features suggesting conservation of this domain fold among τ proteins from other bacterial species (Xun-Cheng et al., 2007).

Bacillus subtilis Cτ interacts with the p33 C-terminal domain of *G. stearothermophilus* DnaB (Haroniti et al., 2004), whereas *G. stearothermophilus* DnaG appears to interact via its C-terminal p16 domain mainly with the p17 N-terminal domain of DnaB (Bird et al., 2000; Bailey et al., 2007). It is reasonable to assume that the interaction interfaces of τ and DnaG with DnaB do not overlap and a ternary complex would form both in vitro (see below) and in vivo. The in vitro activity of DnaG is modulated directly by an interaction with DnaB (Thirlway and Soultanas, 2006; Chintakayala et al., 2008). However, it is not known whether changes in the τ–DnaB interaction can affect allosterically the activity of DnaG. If this were the case, then it would be significant in the context of the replisome in vivo. Given the prominent structural role of τ in the clamp-loader as well as the interaction of the clamp-loader with DnaB and the core polymerase via τ, and with the SSB via χ (Kelman et al., 1998; Glover and McHenry, 1998; Witte et al., 2003), it is important to reveal whether allosteric events mediated by macromolecular interactions within the replisome may be transmitted via the clamp-loader and DnaB to DnaG.

In order to investigate whether changes in the τ–DnaB interaction can affect allosterically the activity of DnaG, we took advantage of a stable ternary complex formed in vitro by *G. stearothermophilus* (strain NCA1503) DnaB, DnaG and *B. subtilis* (strain 168) τ. *G. stearothermophilus* NCA1503 is more closely related to *B. subtilis* 168 than other *Stearothermophilus* strains. This is evident in the high degree of sequence homology between their DnaB (82% identical, 92% similar) (Bird and Wigley, 1999) and DnaG (53% identical, 72% similar) (Pan et al., 1999) proteins, as well as their τ proteins (60% identical, 72% similar). We show that τ can affect allosterically the in vitro activity of the DnaG primase only in the context of a ternary DnaG–DnaB-τ complex. Although native τ does not affect primer synthesis, a mutant τ protein (A550V) isolated by yeast two-hybrid screening with somewhat altered ability to interact with DnaB stimulates the in vitro primer synthesis by DnaG in the ternary complex. Truncation of the last 18 C-terminal residues of τ elicits a primase-stimulatory ability that appears to be suppressed in the native τ. Although this region of Cτ is not involved directly in the interaction with the replicative helicase, it may act as a functional gateway for the allosteric regulation of primase function by replisomal components that interact with τ.

Results

DnaB, DnaG and τ form a stable ternary complex in vitro

The *G. stearothermophilus* DnaB and DnaG proteins form a stable complex in vitro (Bird et al., 2000; Thirlway et al., 2004) with the activities of both proteins modulated by distinct but overlapping networks of interacting interfaces (Thirlway and Soultanas, 2006; Chintakayala et al., 2008). DnaB stimulates primer synthesis and modulates primer sizes in the DnaB–DnaG complex in vitro. The *B. subtilis* 168 and *G. stearothermophilus* NCA1503 strains are closely related. Their τ and DnaB proteins, respectively, form a stable $DnaB_6$–τ_5 complex in vitro (Haroniti et al., 2003; 2004). As there is no detectable interaction between τ and DnaG, while DnaB interacts with both of these proteins, an important question to answer is whether a stable ternary complex can form by mixing purified proteins in vitro.

Using size exclusion chromatography we established that a ternary complex can be formed with DnaB, τ and either DnaG or its DnaB-interacting C-terminal domain known as p16 (Figure 1). Ternary complexes were resolved by gel filtration and samples from the eluted peaks analysed by SDS-PAGE. The results confirmed the formation of the ternary complexes τ–DnaB–p16 (Figure 1A) and τ–DnaB−DnaG (Figure 1B) in the early peaks. No stable complex was detected between τ (1.15 μM pentamer) and DnaG (1.434 μM monomer) (Figure 1C) or τ (3.28 μM pentamer) and p16 (8.22 μM monomer) (Figure 1D). As expected, the DnaB–p16 complex was readily formed (Figure 1E). In order to force sequestration of all the DnaB hexamer into the ternary complex, the experiment shown in Figure 1A was carried out with 2.4 and 6 molar excess of τ pentamer and p16 monomer, respectively, over the DnaB hexamer. The τ–DnaB–p16 complex was detected in the early peak eluted from the Superose 6 HR 10/30 column corresponding to lanes 1–5 in the SDS-PAGE gel, followed by the overlapping τ pentamer lanes 4–6 and the free excess p16 lane 7. By comparison, the experiment shown in Figure 1B was carried out with 2.4 and 3 molar excess of τ pentamer and DnaG monomer, respectively, over the DnaB hexamer. In this case the τ–DnaB−DnaG complex was detected again early in lanes 1–4, followed by the excess τ lanes 5–8. This time no free DnaG was appar-

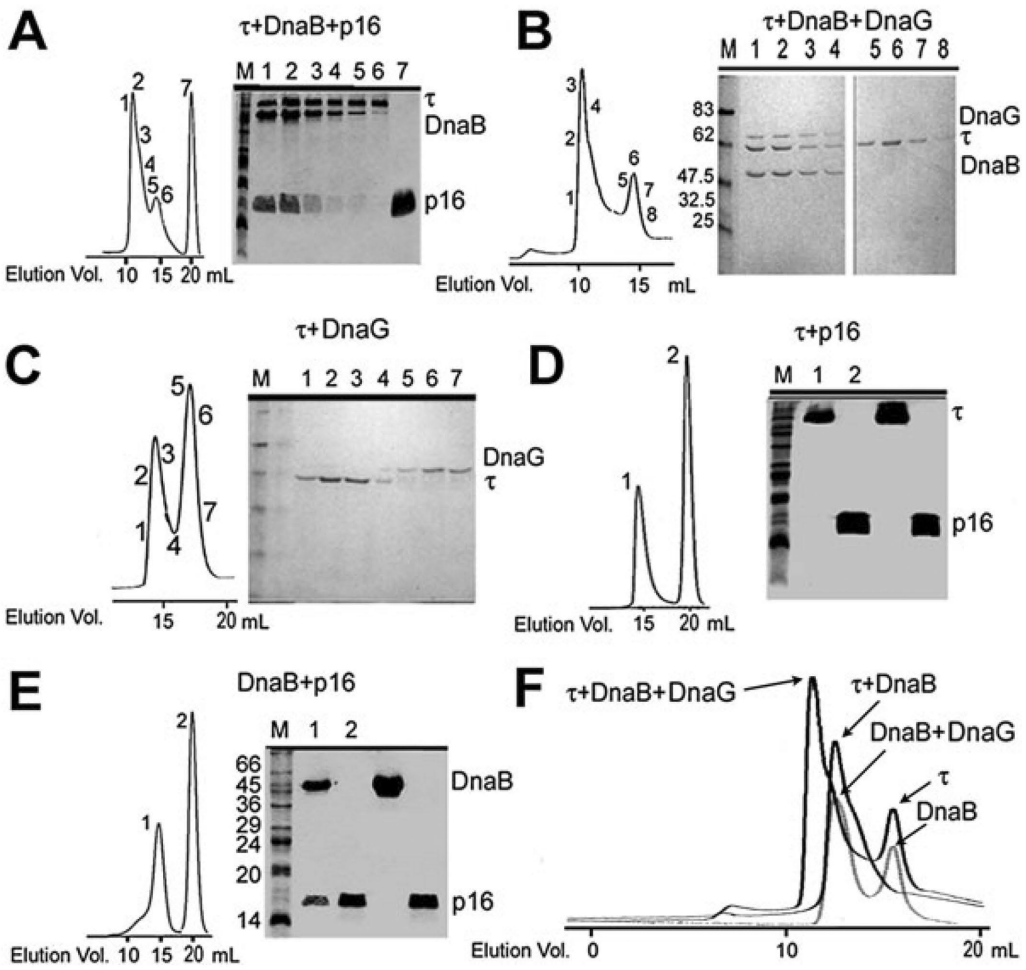

Figure 1. Formation of ternary clamp-loader (τ)-helicase (DnaB)-primase (DnaG) complex.

A. Formation of the τ_5–$DnaB_6$–$p16_3$ ternary complex. DnaB 1.15 μM hexamers were mixed with 3.28 μM τ pentamers and 8.22 μM p16 monomers and the sample was resolved by gel filtration through a Superose 6 HR 10/30 column. The ternary complex is in the early peak, positions marked by numbers 1–5, followed by the overlapping τ pentamer lanes 4–6 and the unbound excess p16 lane 7. Lane M shows the molecular weight markers as indicated in (E.)

B. Formation of the τ_5–$DnaB_6$–$DnaG_3$ ternary complex. Proteins DnaB 0.478 μM hexamers, 1.15 μM τ pentamers and 1.434 μM DnaG monomers were mixed and the sample resolved through a Superose 6 HR 10/30 column. The ternary complex is in the early peak, positions 1–4 along the elution profile, while excess unbound τ is in the late peak positions 5–8. All of the DnaG is sequestered in the ternary complex. Molecular weight markers are shown in lane M.

C. No binary complex is formed between τ (1.15 μM pentamers) and DnaG (1.434 μM monomers).

D. No binary complex is formed between τ (3.28 μM pentamers) and p16 (8.22 μM monomers).

E. A binary $DnaB_6$–$p16_3$ complex is formed between DnaB (1.15 μM hexamers) and p16 (8.22 μM monomers). In all the gels shown the positions along the elution profiles indicated by numbers correspond to the equivalent lane numbers of the gels.

F. Comparative superposition of gel filtration traces from the Superose 6 HR 10/30 column for the ternary and binary complexes as well as the τ pentamer and DnaB hexamer. The ternary complex is clearly resolved from the binary complexes, which in turn are clearly resolved from the τ and DnaB oligomeric proteins.

ent as all was sequestered in the ternary complex. These data are consistent with the presumed correct stoichiometry for the ternary complexes τ_5–$DnaB_6$–$DnaG_3$ and τ_5–$DnaB_6$–$p16_3$. The formation of the ternary complex is clearly visible in Figure 1F where the position of the τ_5–$DnaB_6$–$p16_3$ ternary complex (817 kDa) peak is at ~11 ml compared with ~12.3 ml for the τ_5–$DnaB_6$ complex (615 kDa) peak. The latter cannot be resolved clearly from the $DnaB_6$–$DnaG_3$ complex (504 kDa), which is also at ~12.3 ml. Both binary complexes can be clearly resolved from the τ pentamer and DnaB hexamer, which in turn cannot be resolved from each other.

τ partially inhibits the DnaG-mediated stimulation of the helicase, but not the ATPase activity

DnaG stimulates the ATPase and helicase activities of DnaB in vitro (Bird et al., 2000; Thirlway et al., 2004) whereas *B. subtilis*τ inhibits the helicase activity of *G. stearothermophilus* DnaB in a concentration-dependent

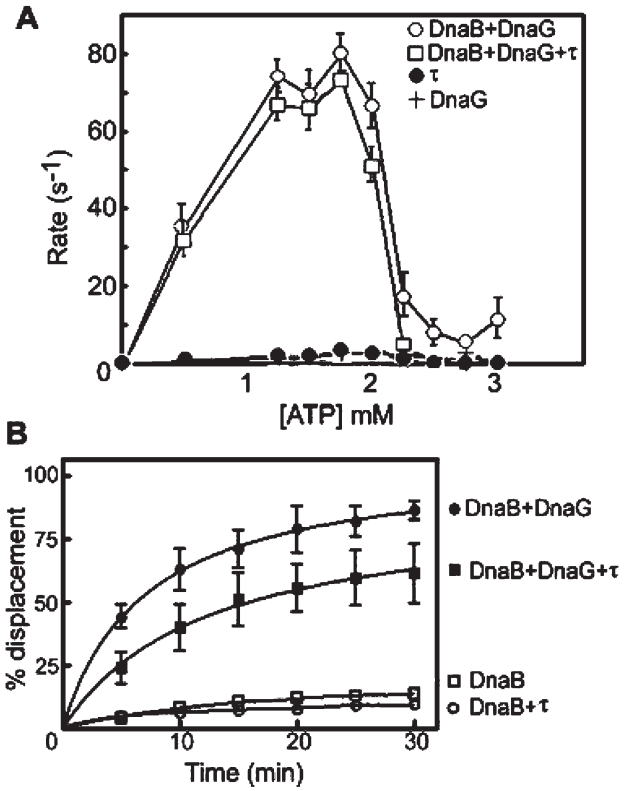

Figure 2. *τ* partially inhibits the DnaG-mediated stimulation of the helicase but not the ATPase activity in the ternary complex.

A. The ATPase steady state turnover rate of the DnaB as a function of time was examined in the presence of DnaG and *τ*, as indicated. The presence of *τ* does not affect the ATPase activity of DnaB in the ternary complex. DnaG and *τ* have no detectable ATPase activity.

B. *τ* partially inhibits the DnaG-mediated stimulation of the helicase activity in the ternary complex. The helicase activity of DnaB in the absence or presence of DnaG and *τ* was examined by in vitro helicase assays. Reactions were carried out with 37.5 nM (hexamer) DnaB in the presence or absence of 675 nM (monomer) DnaG and 37.5 nM (pentamer) *τ*, as indicated. Control reactions of 37.5 nM (hexamer) DnaB in the presence of 675 nM (monomer) DnaG and also with 37.5 nM (pentamer) are shown for comparison. *τ* does not affect the activity of DnaB but appears to partially inhibit the DnaG-mediated stimulatory effect of DnaG in the ternary complex.

manner and does not affect its ATPase activity in vitro (Haroniti et al., 2004). *G. stearothermophilus* DnaB exhibits a non-linear ATPase activity profile with increasing [ATP] (Bird et al., 2000; Soultanas and Wigley, 2002). Although DnaG stimulates the DnaB ATPase activity, it is not known whether *τ* can exert an additional effect in the ternary complex. In order to investigate this we carried out comparative in vitro ATPase assays, as described in **Experimental Procedures**. As expected, DnaG stimulated the ATPase activity of DnaB reaching a maximum level at approximately 1.8 mM [ATP], whereas at higher [ATP] the characteristic inhibition of ATPase activity was apparent (Figure 2A and Bird et al., 2000). The presence of *τ* had no effect on the activity profiles of DnaB in the ternary complex (Figure 2A). From these data we conclude that under our experimental conditions *τ* does not affect the ATPase activity of the DnaB protein in the ternary complex.

The effect of *τ* on the DnaG-mediated stimulation of the helicase activity was then examined with in vitro helicase assays. We found that under our experimental conditions *τ* partially inhibited the stimulatory effect of DnaG on the helicase activity of DnaB (Figure 2B). It has been shown before that *τ* inhibits DnaB in a concentration-dependent manner (Haroniti et al., 2004). Our data suggest that this inhibitory effect is also preserved in the ternary complex (Figure 2B). However, it is still not known whether *τ* can affect the DnaG activity in the ternary complex. Although *τ* and DnaG do not interact directly, it may still be that an allosteric effect is mediated from *τ* to DnaG via DnaB. In order to investigate this possibility we set out first, to isolate *τ* mutants defective in their interaction with DnaB and second, to compare their effects with that of wild-type *τ* using an in vitro primer synthesis assay by DnaG. As C*τ* is directly involved in the *τ*–DnaB interaction we targeted mutations within this domain.

Construction and screening of a mutagenic library for the Cτ

In order to identify amino acid residues within the C*τ* that participate in the interaction with the helicase DnaB, a mutagenic yeast two-hybrid library was constructed by random PCR mutagenesis and screened as described in **Experimental Procedures**. Several white and light blue colonies appeared in our primary screen, indicating no interaction and weak interaction respectively (Figure 3A). Twenty white and five light blue clones were selected for further analysis. Following verification by secondary screening (Figure 3B) as described in **Experimental Procedures**, pACT2 plasmids carrying randomly mutated versions of the C*τ* were isolated from all 25 colonies and then sequenced individually to identify the mutations. Twenty clones that gave a white colour in our primary and secondary screens, indicating no interaction, had either an insertion or a deletion in the coding region of C*τ*, thus explaining the lack of interaction with DnaB. The five light blue colonies had collectively six mutations that led to a change in the coding sequence of C*τ* (F473L, C483S, G492R, L510S, W519R and A550V). The mutated amino acid residues are shown in Figure 3C. The fact that mutating these amino acid residues has affected the ability of C*τ* to interact with DnaB implies that they may be directly involved in the interaction interface or may have altered the overall folding of C*τ*, thus indirectly affecting its interaction with DnaB.

All six single-point mutations were constructed in the full-length *τ* as described in **Experimental Procedures**. The mutant proteins were overexpressed in BL21

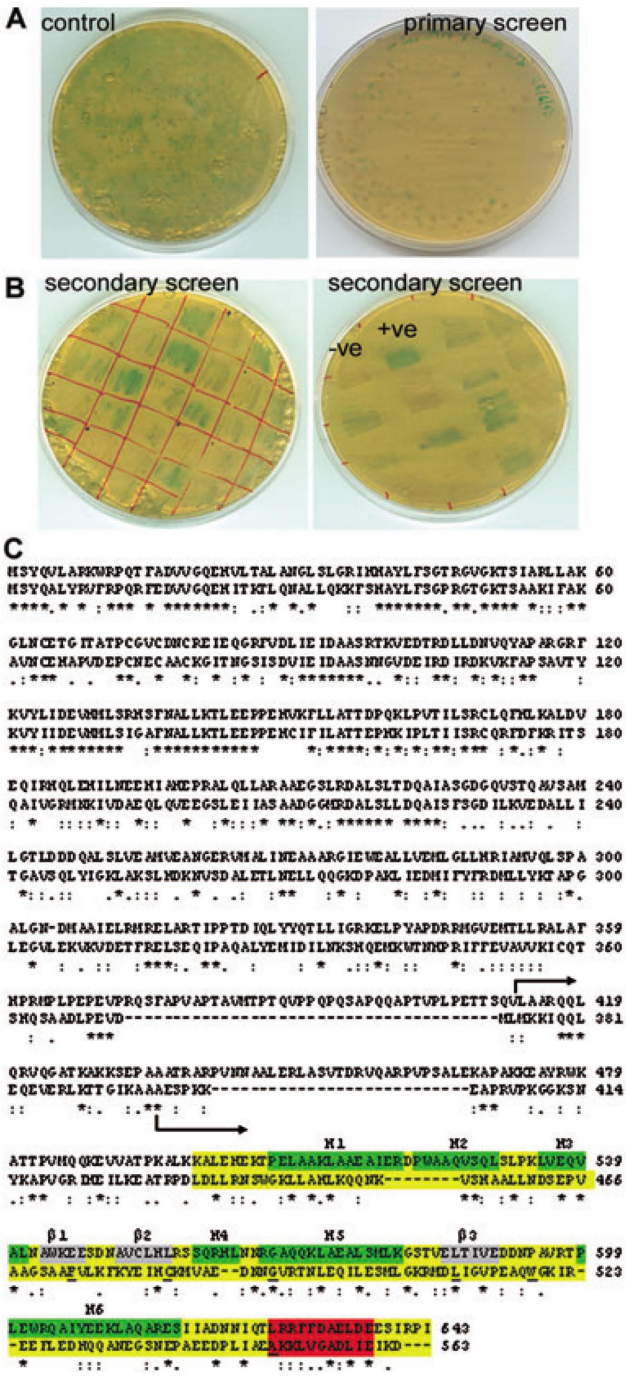

Figure 3. Screening of a mutagenic Cτ library by yeast two-hybrid.

A. Typical results from the primary screening of the mutagenic Cτ library. Yeast colonies carrying wild-type (left) and randomly mutated (right) Cτ clones obtained by the Xgal overlay assay from a primary screen are shown. All colonies are blue in the control plate, indicating interaction with DnaB, while several white and weak blue colonies are visible in the screen indicating defective interactions.

B. Several white and light blue colonies were streaked on new plates and subjected to secondary screening by the Xgal overlay assay. Positive and negative controls are indicated. The strength of the colour represents an estimate of the strength of the DnaB–τ interaction.

C. A clustaw sequence alignment of *E. coli* (top) and *B. subtilis* (bottom) τ proteins. Identical residues are marked by asterisks and similar residues with one or two dots depending on the degree of similarity. The sequence of the *E. coli* Cτ domain whose NMR structure has been solved (Xun-Cheng et al., 2007) is coloured, with helices H1-H6 green and β-sheets β1-β3 grey and labelled appropriately. The equivalent Cτ regions comprising domains IVa (residues 413–496) and V (residues 497–643) in *E. coli* and domain III (residues 397–563) in *B. subtilis* (Haroniti et al., 2004) are indicated by arrows. Sequencing of plasmids obtained from light blue colonies, isolated from the yeast two-hybrid screen, identified six mutations F473L, C483S, G492R, L510S, W519R and A550V. These are indicated along the sequence of the *B. subtilis* Cτ in bold/underlined letters. The C-terminal unstructured region with good conservation containing the A550 residue is highlighted in red.

(DE3) *E. coli*, but only A550V gave us soluble protein (Figure S1). Various attempts to solubilize the other five mutant proteins by altering the expression conditions and solubilization buffers were unsuccessful (data not shown). We concluded that all of these mutations, with the exception of A550V, have resulted in somewhat misfolded τ proteins affecting their solubility properties hence their defective interactions with DnaB indicated by the yeast two-hybrid screen.

A550V forms a ternary complex with DnaB and DnaG

The A550V mutant protein was purified and shown by analytical gel filtration to still form binary A550V–DnaB and ternary A550V–DnaB–DnaG complexes (Figure 4A and B). The τ–DnaB and A550V–DnaB complexes were formed and eluted through the Superose 6 10/30 column at 0.478 μM τ or A550V pentamer and 0.478 μM DnaB hexamer, consistent with the τ_5–$DnaB_6$ and $A550V_5$–$DnaB_6$ stoichiometries (Figure 4A). The ternary A550V–DnaB–DnaG complex was formed at 0.478 μM DnaB hexamer, 1.434 μM DnaG monomer and 1.15 μM A550V pentamer (Figure 4B). All of the DnaG was sequestered into the ternary complex (Figure 4B lanes 1–4) whereas the excess A550V pentamer was present in the late peak (Figure 4B lanes 5–7). This is again consistent with an $A550V_5$–$DnaB_6$–$DnaG_3$ stoichiometry. As expected, our data show that the A550V mutation did not abolish the τ–DnaB interaction. The defect is likely to be subtle and detectable by our sensitive yeast two-hybrid screen (evident by the light blue coloration) but not by gel filtration. In order to establish whether this is the case we investigated whether A550V has any functional effects on the DnaB helicase activity. Our data show that A550V partially inhibits the stimulatory effect of DnaG on DnaB (Figure 4C). When compared with the wild-type τ the effect observed for A550V is marginally weaker, implying a slightly altered protein–protein interaction (compare Figs 2B and 4C). The effects of both wild-type τ and A550V proteins on the activity of DnaG were then investigated in the context of the ternary complex.

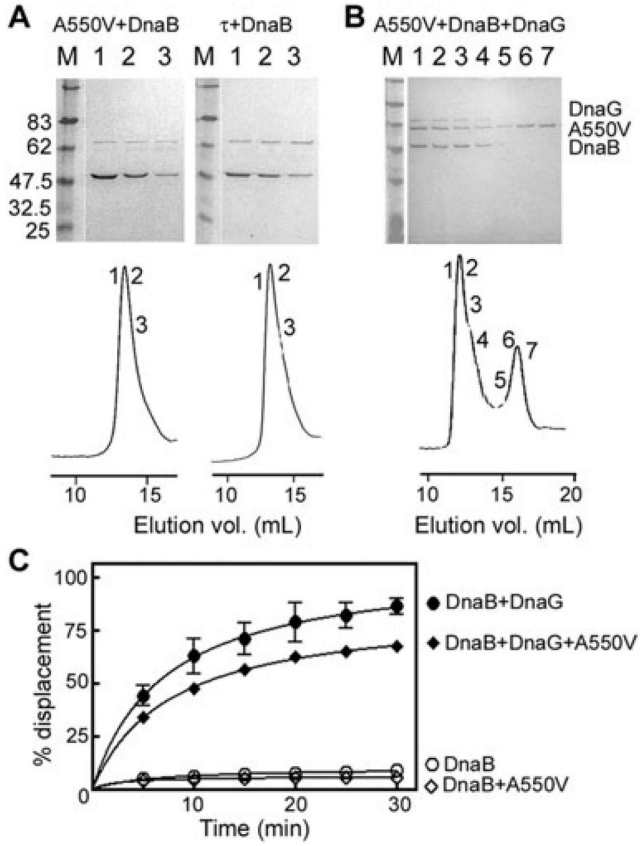

Figure 4. A550V interacts with DnaB and partially inhibits the stimulatory effect of DnaG.

A. The complexes A550V–DnaB and τ–DnaB were isolated by gel filtration through a Superose 6 10/30 column, as indicated. Complexes were formed by mixing 0.478 μM DnaB hexamer with equimolar concentrations of A550V or τ pentamers, as described in **Experimental Procedures**. Samples from the fractions equivalent to the positions marked by the numbers along the peaks were analysed by SDS-PAGE to verify the presence of proteins in the complexes. The numbers of the lanes in the gel correspond to the equivalent numbers indicating the positions of the fractions along the peaks. Molecular weight markers are shown in lane M for all the gels.

B. Formation of the $A550V_5$–$DnaB_6$–$DnaG_3$ ternary complex by mixing 0.478 μM DnaB hexamers with 1.434 μM DnaG monomers and 1.15 μM A550V pentamers. The ternary complex is in the early peak positions 1–4 while excess A550V is in the late peak positions 5–7. The positions indicated along the elution profiles are equivalent to the same lane numbers shown in the gel.

C. A550V (37.5 nM pentamer) partially inhibits the stimulatory effect of DnaG (675 nM monomer) on DnaB (37.5 nM hexamer) helicase activity in the ternary complex. The inhibitory effect is marginally smaller than that observed for the wild-type τ in Figure 2B. In the context of the binary complex A550V has no significant effect on the DnaB helicase activity. The error bars in the ternary complex and the control DnaB and DnaB + A550V data are too small to be visible.

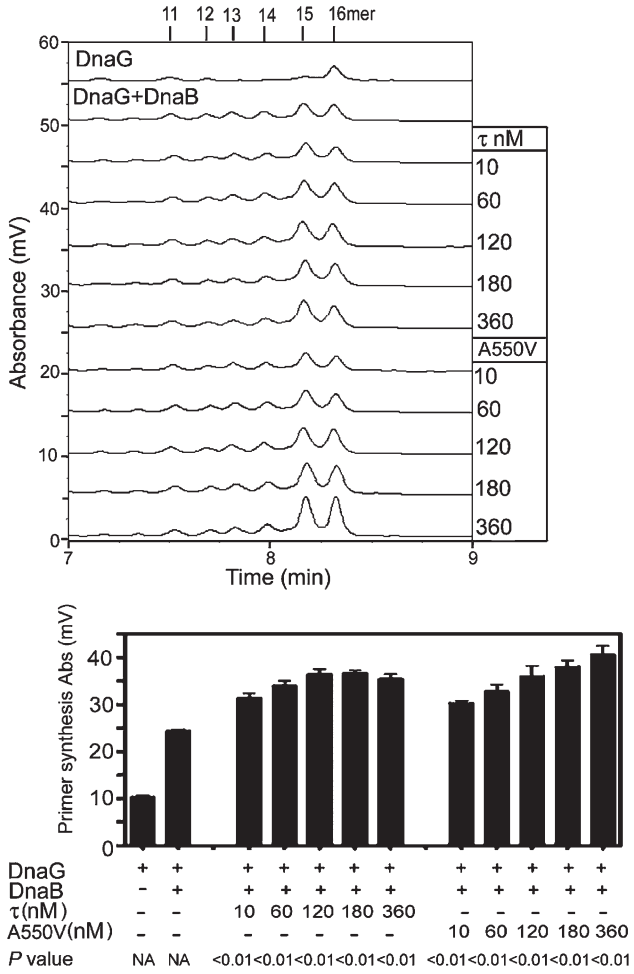

Figure 5. τ stimulates primer synthesis in the ternary complex. Representative chromatograms from denaturing HPLC analysis of RNA primers produced by DnaG in the presence of DnaB, as a function of wild-type τ or mutant A550V concentrations. The primase activity reactions were incubated for 1 h at 37°C with 720 nM DnaG, without or with 60 nM (hexamer) DnaB, 2 μM ssDNA 23-mer template containing the preferred initiation trinucleotide 5′-d(CTA), 400 μM of each NTP and wild-type τ or mutant A550 at the monomer concentrations specified. Three independent experiments were carried out. Bar graphs and P-values from the statistical analysis of the data are also shown.

τ stimulates primer synthesis by DnaG only in the ternary complex while the A550V mutant stimulates DnaG to a greater extent in a concentration-dependent manner

Primer synthesis by the ternary complex was examined, as described in **Experimental Procedures**, at 720 nM DnaG, 60 nM DnaB (hexamer) and increasing concentrations of τ or mutant A550V proteins, using a 23-mer oligonucleotide containing the preferred initiation oligonucleotide 5′-d(CTA) (Thirlway and Soultanas, 2006). Three independent experiments were performed and the average data showed that, under our experimental conditions at 37°C in the control reactions with the DnaG–DnaB complex, DnaG synthesized a range of smaller primers and was stimulated twofold by DnaB (Figure 5). In the presence of 60–360 nM τ (monomer), primer yield increased consistently by 17% whereas in the presence of the A550V mutant at the same concentrations there was a marked progressive increase in primer yield culminating in a 30% increase at the highest concentration of A550V (360 nM, monomer) (Figure 5). This

is equivalent to approximately twofold greater stimulation of the primase activity compared with that obtained by τ at the same concentration (Figure 5). This stimulatory effect is only observed in the context of the ternary complex as τ and A550V fail to exhibit a stimulatory effect on DnaG alone Figure 6). We conclude that both A550V and τ can additionally stimulate the DnaG in the ternary complex. Stimulation by A550V is concentration-dependent and greater than that observed by τ. As the interaction of τ with DnaB has somewhat changed by the A550V mutation we further conclude that changes in the clamp-loader–helicase interaction can affect primer synthesis in vitro.

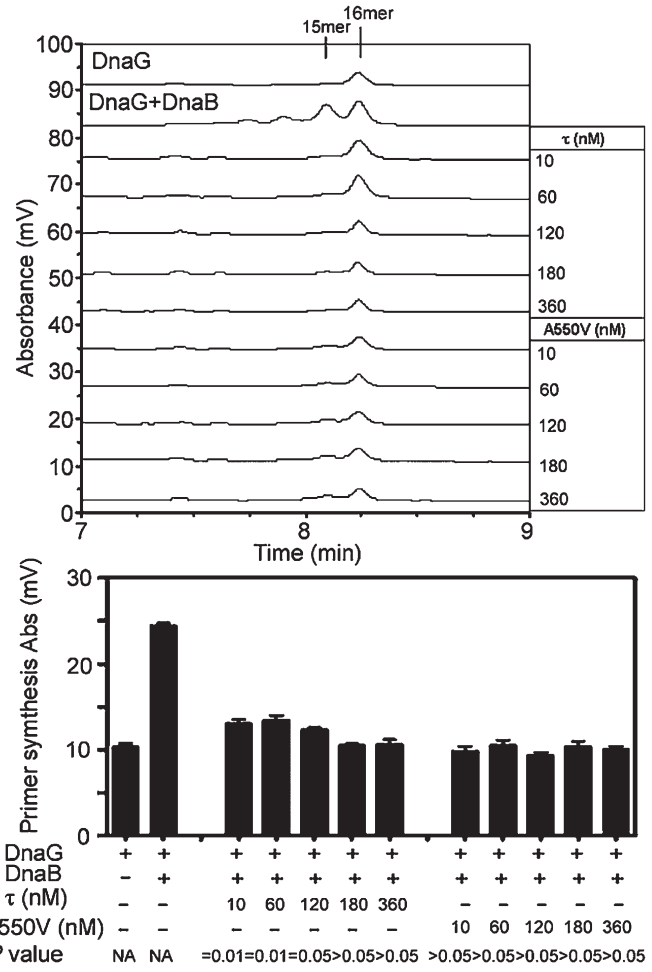

Figure 6. τ does not affect primer synthesis by DnaG in the absence of DnaB. Representative chromatograms from denaturing HPLC analysis of RNA primers produced by DnaG in the absence of DnaB, as a function of wild-type τ or mutant A550V concentrations, as indicated. Experimental conditions are as described in Figure 5. A control reaction to show the effect of DnaB (60 nM monomer) is also shown. Three independent experiments were carried out. Bar graphs and P-values from the statistical analysis of the data are also shown.

Truncation of 18-amino-acid residues at the C-terminus of τ does not abolish binding to DnaB but causes stimulation of primer synthesis in the ternary complex

The solution structure of the C-terminal domain IVa-V of the *E. coli*τ, which is equivalent to Cτ, has revealed a unique fold with a highly solvent exposed and mobile 30-residue segment at the extreme C-terminus that interacts with the α subunit of the DNA polymerase holoenzyme III (Jergic et al., 2007; Xun-Cheng et al., 2007). Despite the weak sequence conservation within the Cτ domains in different bacterial species, certain features suggest structural conservation. However, significant homology is apparent in the flexible extreme C-terminus of τ from different bacteria (Xun-Cheng et al., 2007), including the *E. coli* and *B. subtilis* Cτ (Figure 3C). Interestingly, residue A550 is situated within this region although it is not strictly conserved. In order to establish whether this region is involved directly in the interaction with DnaB or whether the effect we have observed is a result of an indirect functional perturbation, we deleted the last 18 residues at the C-terminus to construct a truncated version of τ (Δτ18). Δτ18 was still able to interact with DnaB in binary $\Delta\tau18_5$–$DnaB_6$ or ternary $\Delta\tau18_5$–$DnaB_6$–$DnaG_3$ complexes that were isolated by gel filtration in a similar manner to native τ (Figure 7A and B). The truncated Δτ18 behaved more like the A550V mutant protein and increased primase activity significantly beyond the DnaB-mediated stimulation (Figure 7C) while its suppression of the DnaG-mediated stimulation of DnaB was better than A550V and native τ (Figure 7D). DnaB stimulated DnaG primase activity by twofold in the control reactions and the presence of Δτ18 at the lower concentrations of 10 and 60 nM did not alter the amount of RNA polymers produced by DnaB-stimulated primase (Figure 7A). However, at higher concentrations of Δτ18, primer synthesis increased significantly. At 120, 180 and 360 nM of Δτ18, a 24%, 38% and 28% increase in the amount of primers was obtained, respectively, with saturation occurring at 180 nM (Figure 7C). When comparing the amount of primers synthesized in the presence of either truncated or full-length τ at these higher concentrations, the yield of primers produced by the $\Delta\tau18_5$–$DnaB_6$–$DnaG_3$ complex was almost twofold higher than the amount of RNA polymers synthesized by the τ_5–$DnaB_6$–$DnaG_3$ complex. Therefore, truncation of the last 18 C-terminal residues elicits a primase-stimulatory ability of τ that appears to be suppressed in the native protein. Hence these residues of τ may act as a "functional gateway" for the allosteric regulation of the primase activity by components of the replisome that interact with τ via its C-terminal end.

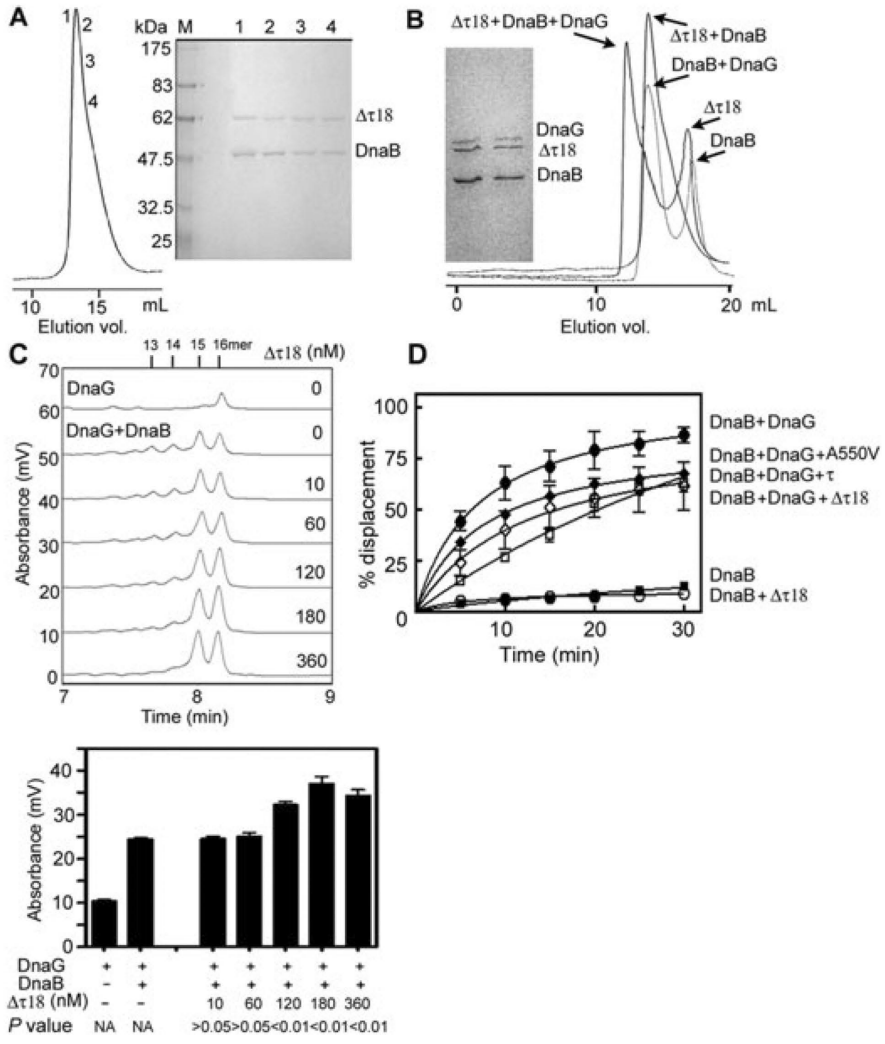

Figure 7. The truncated Δτ18 protein binds to DnaB and stimulates primer synthesis in the ternary complex.

A. Formation of the $\Delta\tau18_5$–$DnaB_6$ complex. Proteins DnaB 0.478 μM hexamers and 0.478 μM Δτ18 pentamers were mixed and the sample resolved through a Superose 6 HR 10/30 column. Both proteins were detected in the complex by SDS-PAGE analysis. The positions 1–4 along the elution profile correspond to the lanes in the gel. Molecular weight markers are shown in lane M.

B. Superposition of the gel filtration traces of binary ($DnaB_6$–$DnaG_3$, $\Delta\tau18_5$–$DnaB_6$) and ternary ($\Delta\tau18_5$–$DnaB_6$–$DnaG_3$) complexes through a Superose 6 HR 10/30 column to demonstrate the formation of the ternary complex with Δτ18. The relevant peaks are indicated by arrows and labelled appropriately. Samples from the peak corresponding to the ternary complex were analysed by SDS-PAGE to verify the presence of the three proteins.

C. Representative chromatograms from denaturing HPLC analysis of RNA primers synthesized by DnaG primase in the presence of DnaB as a function of Δτ18 monomer concentration, as indicated. The primase activity reactions were incubated for 1 h at 37°C with 720 nM DnaG, without or with 60 nM (hexamer) DnaB, 2 μM ssDNA 23-mer template containing the preferred initiation trinucleotide 5′-d(CTA), 400 μM of each NTP and Δτ18 at the monomer concentrations specified. Triplicate reactions in independent experiments were evaluated. Bar graphs and P-values from the statistical analysis of the data are also shown.

D. Δτ18 inhibits the helicase activity in the ternary complex stronger than A550V and native τ. Reactions were carried out with 37.5 nM (hexamer) DnaB in the presence or absence 675 nM (monomer) DnaG and 37.5 nM (pentamer) Δτ18, as indicated. Comparative graphs showing the effects of Δτ18, τ (from Figure 2B) and A550V (from Figure 4C) are presented. The error bars in the DnaB control are too small to see.

Discussion

Allosteric regulation of primer synthesis

Primer synthesis by DnaG is regulated by an interaction with the replicative hexameric DNA helicase DnaB. DnaB modulates the initiation specificity, stimulates primer synthesis and reduces the size of primers synthesized by DnaG (Lu et al., 1996; Bhattacharyya and Griep, 2000; Johnson et al., 2000; Mitkova et al., 2003; Koepsell et al., 2006; Chintakayala et al., 2008). Three DnaG molecules interact with the DnaB hexamer (Bird et al., 2000; Mitkova et al., 2003; Bailey et al., 2007), but other complexes with one or two DnaG molecules per DnaB hexamer have been observed in vitro by atomic force microscopy (Thirlway et al., 2004). Stoichiometric variability has been proposed as a regulatory mechanism for primer synthesis, whereby DnaG molecules bound to the DnaB hexameric ring interact and modulate each others' activities (Thirlway et al., 2004; Corn et al., 2005). However, it is not known whether primer synthesis can be modulated allosterically by macromolecular interactions in the context of an active multi-protein replisome in vivo. One could envisage a scenario whereby other proteins that interact with DnaB may also affect allosterically the activity of DnaG. One such prominent protein is the τ subunit of the clamp-loader that interacts via its Cτ with DnaB. Our data show that while τ cannot affect directly the activity of DnaG, it can stimulate primer synthesis in the DnaG–DnaB–τ ternary complex, in addition to the DnaB-mediated stimulatory effect. An A550V mutation in Cτ affects the interaction of τ with

DnaB and most importantly this change also affects allosterically primer synthesis by DnaG. It is therefore feasible to modulate in vivo primer synthesis allosterically within the replisome via macromolecular protein–protein interactions.

The inhibitory effect of the A550V mutant protein on the helicase activity of the DnaG–DnaB complex is marginally weaker than that of the wild-type τ, but the effect of Δτ18 is stronger, as shown in Figure 7D, and yet both A550V and Δτ18 stimulate primer synthesis more effectively than τ. Therefore, the stimulatory effect by τ on the primase activity in the context of the ternary complex is independent of the helicase activity. Hence we conclude that increased helicase activity is not a prerequisite for increased priming activity. If such a functional mechanism were to operate whereby the primase activity can be stimulated in the lagging strand without the concurrent stimulation of the tightly associated helicase activity, then this could facilitate the co-ordination of slow lagging-strand synthesis with the faster leading-strand synthesis. Data from single-molecule studies of the *E. coli* replisome suggest that modulation of DnaB activity via a direct interaction with DnaG, independent of DnaG activity, slows down leading-strand synthesis by threefold and prevents it from outpacing lagging-strand synthesis (Tanner et al., 2008). Our data suggest that it is also possible that other allosteric interactions through the clamp-loader may alternatively stimulate the primase activity, independently of the helicase activity, to speed up lagging strand synthesis and facilitate co-ordination of DNA replication of the two parental strands.

The observed in vitro inhibitory effect of τ on the helicase activity of DnaB is contradictory with reports that τ-mediated stimulation of DnaB is crucial for the rapid progression of the replisome (Kim et al., 1996). One explanation offered for this is that other replisomal proteins within the replication fork in vivo may alleviate this inhibitory effect observed in vitro (Haroni et al., 2004). Our data show that the primase DnaG on its own cannot alleviate the τ-mediated inhibition of the helicase DnaB in the ternary complex in vitro.

The unstructured C-terminus of τ is not the site of interaction with DnaB but is a functional gateway through which allosteric events can be transmitted to DnaG

All of the DnaB interaction defective mutations identified by our yeast two-hybrid screen were confined within the C-terminal half of the Cτ sequence in a region that would be equivalent to domain V of the *E. coli*τ (Figure 3C). Yet a conserved L381 residue in *B. subtilis*τ in a distant region near the domain III–IVa boundary was previously shown to be important for the interaction of τ with *G. stearothermophilus* DnaB, with a L381A mutation affecting τ oligomerization and the interaction with DnaB (Haroniti et al., 2003). This residue is in a conserved sequence repeat pattern of hydrophobic and hydrophilic residues predicted to be in α helical coiled coil structural motifs that mediate subunit oligomerization of a large number of proteins (Lupas, 1996; Buckhard et al., 2001). They consist of two to five amphipathic α helices that twist around one another to form a supercoil. Their main characteristic feature is a heptad repeat sequence of polar and non-polar residues. The latter constitute the oligomer interaction interface (Figure S2). The stability of the coiled coils is achieved by packing of the non-polar side-chains into a hydrophobic environment and ionic interactions between the residues flanking the core. The L381A mutation is likely to have affected the integrity of this coiled coil (Figure S2) and thus the integrity of the τ oligomer. Therefore, the DnaB interaction defect of the L381A mutation is likely to be an indirect effect explained by local effects on the protein conformation, rather than a direct indication of an interaction interface (Haroniti et al., 2003). The DnaB interaction interface in *E. coli*τ has been mapped to residues 430–496 within domain IVa (Gao and McHenry, 2001a) and from our data with the Δτ18 protein it appears that the DnaB interaction interface of the *B. subtilis*τ is located before the last 18 C-terminal residues. If, however, the extreme C-terminus of *B. subtilis*τ and of other different bacteria is involved in the interaction with the polymerase, as structural and homology data seem to suggest (Xun-Cheng et al., 2007), then this region including A550 may also be a functional gateway to modulate primer synthesis by the bound polymerase, hence coupling the two activities within the replisome. In this model the polymerase α interacts with τ via Cτ and alters the DnaB–τ interaction which in turn affects the activity of DnaG attached to DnaB. This functional association of the polymerase and primase activities may be an important aspect of co-ordinating fast leading-strand and slow lagging-strand synthesis. A tight association of these two activities is manifested clearly in eukaryotes. Typically, yeast, *Drosophila*, mouse and human primases purify as a complex of four proteins that include two primase subunits and two polymerase subunits (Pol α and B), reviewed in Frick and Richardson (2001). Such intimate association is also a reflection of the need to co-ordinate DNA replication with cell cycle control. Phosphorylation events by the cyclin-dependent kinases cyclin A/cdk2 and cyclin E/cdk2 that regulate G1 to S transition target Pol α and B to modulate the activity of the Pol α/primase complex. The absence of a strict cell cycle regulatory mechanism in bacteria has negated the need for direct physical association of the primase and polymerase but essential functional communications between these two activities to co-ordinate leading- and lagging-strand synthesis are still preserved via indirect allosteric interactions within the replisome.

Experimental procedures

Protein purifications

The DnaB, τ, DnaG and p16 proteins were prepared as described elsewhere (Bird et al., 2000; Haroniti et al., 2003). The mutant τ A550V and the Δτ18 proteins were purified exactly as the native τ.

Size exclusion chromatography

The formation of the τ–DnaB–p16 and τ–DnaB−DnaG ternary complexes were isolated by size exclusion chromatography using a Superose 6 HR 10/30 column (GE Healthcare). The column was equilibrated in TED/100 [50 mM Tris pH 7.5, 2 mM EDTA, 1 mM dithiothreitol (DTT), 100 mM NaCl]. In order to obtain the τ–DnaB–p16 complex the proteins were mixed in TED/100 at 1.37 μM DnaB hexamer, 3.28 μM τ pentamer and 8.22 μM p16 monomer incubated on ice for 5–10 min and then loaded directly onto the column. For the τ–DnaB−DnaG complex the proteins were mixed in TED/100 at 0.478 μM DnaB hexamer, 1.15 μM τ pentamer and 1.434 μM DnaG (monomer) at appropriate combinations, incubated on ice for 5–10 min and then loaded directly onto the column. The τ–DnaB complexes were obtained by mixing 0.478 μM DnaB hexamer and an equimolar amount of τ pentamer. Similar experiments were carried out for the A550V–DnaB–DnaG complex. Samples from the eluted peaks were analysed by standard SDS-PAGE to verify the presence or absence of various proteins.

ATPase assays

The steady state ATPase activity of DnaB was measured by linking ATP hydrolysis to the oxidation of NADH as described previously (Bird et al., 2000). ATPase assays were carried out with 10 nM DnaB (hexamer) and 60 nM DnaG (monomers) in the presence or absence of 60 nM τ (pentamer) in 50 mM Tris (pH 7.4), 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM DTT, 2.5 nM single-stranded M13mp18 DNA and varying concentrations of ATP (0, 0.5, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75, and 3.0 mM).

Helicase assays

The DNA substrate for helicase reactions was prepared by radiolabelling the oligonucleotide 5′-GTTATTGCATGAAAGCCCGGCTGACTCTAGAGGATCCCCGGGTACGTATTGCATGAAAGCCCGGCTG-3′ (underlined bases are complementary to a unique sequence on M13mp18 ssDNA) at the 5′-end using [γ-^{32}P]-ATP and T4 polynucleotide kinase (NEB). The radiolabelled oligonucleotide was then annealed to single-stranded M13mp18 DNA to produce a 3′-5′-tailed DNA substrate. One molecule of DNA substrate is defined as one molecule of single-stranded M13mp18 DNA with one molecule of oligonucleotide annealed to it. Reactions were carried out at 37°C in 50 mM Tris (pH 7.4), 50 mM NaCl, 12.5 mM $MgCl_2$, 2.5 mM ATP, 1 mM DTT, 0.5 nM DNA substrate. Helicase reactions were carried out with 37.5 nM (hexamer) DnaB in the presence or absence of 675 nM (monomer) DnaG and 37.5 nM (pentamer) τ. The appropriate proteins were mixed separately and incubated at room temperature for 10 min to enhance complex formation. Reactions were initiated by the addition of ATP and 20 μl samples were removed at appropriate time intervals, the reaction was terminated by the addition of 5 μl stop buffer (0.4% w/v SDS, 40 mM EDTA, 8% v/v glycerol, 0.1% w/v bromophenol blue) and stored briefly at 4°C, prior to electrophoresis through a non-denaturing 8% v/v polyacrylamide gel in 1× TBE. Gels were dried under vacuum. Imaging and quantitative analysis was carried out by a Molecular Imager FX (Bio-Rad) and associated software. All reactions were carried out in duplicate and data were plotted as percentage of radiolabelled oligonucleotide displaced from the single-stranded M13 DNA versus time.

Yeast two hybrid

The yeast two-hybrid experiments were carried out with the AH109 yeast strain (James et al., 1996) using the MATCHMAKER two-hybrid system from Clontech, as described elsewhere (Haroniti et al., 2004). Briefly, the *dnaB* gene was cloned in the NcoI-SalI sites of pAS2-1 (Harper et al., 1993), while the *dnaX* gene fragment that codes for the various domains of τ including the Cτ and its randomly mutagenized versions were cloned in NcoI-XhoI sites of pACT2 (Li et al., 1994). Screening by yeast two-hybrid was carried out by the agarose Xgal overlay method (Dumay et al., 1999). All yeast transformations were carried out by electroporation, as described elsewhere (Becker and Guarente, 1991). Plasmids were isolated from yeast cultures as described in the "Yeast Protocols" handbook of Clontech.

Construction of a mutagenic library for the Cτ

A mutagenic library of the Cτ was constructed by random PCR mutagenesis (Fromant et al., 1995) using as template the pACT2 vector carrying the corresponding Cτ region of the dnaX gene (Haroniti et al., 2004). Based upon the sequence of the pACT2 vector two primers were used: forward 5′-CTATTCGATGATGAAGATACCCCACCAAAC-3′ and reverse 5′-GAGATGGTGCACGATGCACAGTTGAAG-3′. Eight PCR reactions were carried out each in a total volume of 50 μl containing 0.5 μM forward and reverse primers, 2.8 mM dNTP mix, 0.1 mM $MnCl_2$, 0.8 mM $MgCl_2$, 250 ng template, 2.5 units Taq polymerase, 10 mM Tris-HCl, pH 8.3 and 50 mM KCl. The reactions were subjected to one cycle of 4 min 94°C, 5 min 55°C, 2 min 72°C and to 30 cycles of 30 s 94°C, 1 min 55°C and 2 min 72°C. The PCR products were resolved through a 1% w/v agarose gel in 1× TBE, extracted from the gel using the QIAGEN gel extraction kit, digested with NcoI-XhoI overnight and then cloned into pACT2 and transformed into electro-

competent *E. coli* XL1 Blue cells. All the transformants from ampicillin selective plates were pooled and used to inoculate an overnight *E. coli* XL1 Blue cell culture. A preparation of the library of mutagenic plasmids was isolated the following day using the midi-prep plasmid QIAGEN kit. This plasmid preparation was then used in yeast two-hybrid screens.

Yeast two-hybrid screening of the mutagenic library

The pACT2 library carrying random mutants of the C*τ* was transformed into yeast containing pAS2-1 carrying the *dnaB* gene. Yeasts were then plated on media lacking leucine and tryptophan to select for colonies containing both plasmids. The primary screening was carried out by the agarose Xgal overlay method. As the strength of the colony coloration is an estimate of the strength of an interaction white and light blue colonies were targeted for further analysis. Appropriate colonies were randomly isolated and subjected through a secondary screen by streaking on new selective media and checking again by the agarose Xgal overlay method. Following verification in the secondary screen, the pACT2 plasmids were isolated and sequenced individually. All 20 clones that gave a white colour in our primary and secondary screens, thus indicating no interaction, had either an insertion or a deletion in the coding region of C*τ*.

Site-directed mutagenesis

Site-directed mutagenesis was carried out using the QuickChange II-E Site Mutagenesis Kit (Stratagene), using the pET28-*dnaX* plasmid (Haroniti et al., 2003) and appropriate mutagenic oligonucleotides (Figure S3.). All the correct mutations and the absence of spurious mutations were verified by sequencing.

In vitro primer synthesis assay

Ribonucleoside triphosphates (NTPs) were obtained from Promega (Madison, WI). Magnesium acetate, potassium glutamate, HEPES and DTT were from Sigma-Aldrich (St Louis, MO). The Sephadex G-25 microspin columns were purchased from Amersham (Piscataway, NJ). Primer lengths and quantities were measured using a WAVE HPLC Nucleic Acid Fragment Analysis System with a DNA Sep HPLC column, buffer A (0.1 M triethylammonium acetate, pH 7.0) and buffer B (0.1 M triethylammonium acetate, 25% acetonitrile v/v) that were obtained from Transgenomic (Omaha, NE).

The 23-mer oligonucleotide used in this study consisted of the sequence 5′-CAGA$(CA)_5$XYZ $(CA)_3$-3′ where XYZ was CTA, the preferred trinucleotide for *G. stearothermophilus* DnaG (Thirlway and Soultanas, 2006). This single-stranded DNA template was generated by Integrated DNA Technologies (Coralville, IA) and contained a 3′-C3 spacer that is required to prevent primase from elongating from a stabilized 3′-hairpin (Bhattacharyya and Griep, 2000; Koepsell et al., 2004). Purification of the ssDNA template was performed using urea-PAGE, UV-shadowing and elution of the oligonucleotide into Tris-EDTA buffer. The concentration was determined using spectrophotometry at 260 nm with the extinction coefficient of 229 600 $M^{-1}cm^{-1}$.

The RNA primer synthesis reactions were performed in 100 µl nuclease-free water containing 50 mM HEPES, 100 mM potassium glutamate (pH 7.5), 10 mM DTT, 10 mM magnesium acetate, 400 µM of each NTP, 2 µM of the 23-mer CTA template and 720 nM of primase. The reactions were incubated either without or with 60 nM (hexamer) DnaB and with the wild-type *τ* or mutant *τ* (A550) protein at the monomer concentrations indicated. The primer synthesis assays were performed at the temperature previously determined to be optimum for maximum helicase stimulation of *G. stearothermophilus* DnaG activity, specifically 37°C (M.A. Larson, unpublished). After incubation for 1 h, samples were desalted in a Sephadex G-25 spin column, dried using a speed vacuum, and the pellet was suspended in 10 µl of nuclease-free water.

HPLC under thermally denaturing conditions at 80°C was used to analyse 8 µl of each sample as previously described (Koepsell et al., 2004). The primers synthesized in the reactions were eluted with a flow rate of 0.9 ml min^{-1} using a gradient of 0–8.8% (v/v) acetonitrile over 16 min, for optimal separation of RNA polymers and ssDNA template peaks. Both the primers and ssDNA templates were detected by UV absorbance at 260 nm. Retention times of the single-stranded oligoribonucleotide and oligodeoxyribonucleotide standards were correlated to the retention times of the appropriate oligonucleotide standard to confirm composition and length. The RNA standards utilized in this study consisted of the sequence 5′-AGUGUGUGUGUG-3′ (12-mer), 5′-AGUGUGUGUGUGUC-3′ (14-mer) and 5′-AGUGUGUGUGUGUCUG-3′ (16-mer). The moles of primers were quantified by using the template as an internal standard, as previously described (Koepsell et al., 2008). This method normalized the variability introduced to the system during sample preparation and injection into the HPLC column. All primer synthesis reactions were carried out in triplicate and data are presented as the mean ± standard error from triplicates. Statistical analyses were carried out by the two-tailed unpaired Student t-test with 95% confidence intervals using Prism (version 5) software (GraphPad Software Inc.). Differences with a P-value of < 0.05 are generally considered significant but, in order to be more stringent, P-values of < 0.01 were considered significant for these experiments.

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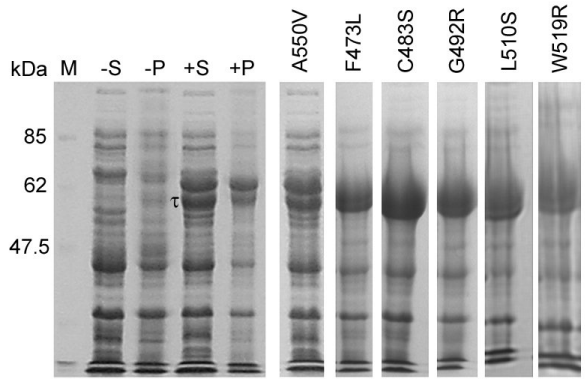
[Supplementary Figures 1S, 2S, and 3S follow.]

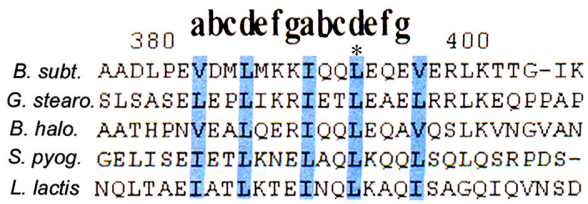
Supplementary information

Supplementary Fig. 1s



SDS-PAGE analysis of soluble (S) and insoluble (P) extracts, in the presence (+) and absence (-) of IPTG, from BL21 *E. coli* (DE3) cells to test for over-expression of wild type τ and mutant proteins as indicated. The wild type τ and the mutant A550V proteins were expressed in the soluble fraction while the other mutant proteins were in the insoluble fractions, as indicated. Molecular weight markers are shown in lane M.

Supplementary Fig. 2s



The L381 residue in the *B. subtilis* sequence found previously to be important for oligomerisation and interaction with the DnaB (Haroniti *et al.*, 2003), is marked by an asterisk. A clustaw alignment of this sequence region between different *dnaX* sequences from gram positive bacteria *B. subtilis, G. stearothermophilus, Bacillus halodurans, Streptococcus pyogenes, Lactococcus lactis*. L381 is part of a heptad sequence pattern of hydrophobic (a, d) and hydrophilic (b, c, e, f, g) residues characteristic of a coiled coil structural motif.

Supplementary Fig. 3s

dnaX oligonucleotide sequences

Mutant F473L

Direct FA 5’-GGCTGCCGGATCGGCGGCACTTGTTCTGAAATTCAAA-3’
Reverse FA 5’-TTTGAATTTCAGAACAAGTGCCGCCGATCCGGCAGCC-3’

Mutant C483S

Direct CA

5’-GGCATTTGTTCTGAAATTCAAATATGAAATTCATTCTAAAATGGTCGCTGAGGATAAC-3’

Reverse CA

5’-GTTATCCTCAGCGACCATTTTAGAATGAATTTCATAATTTGAATTTCAGAACAAATGCC-3’

Mutant G492R

Direct GA 5’-CGCTGAGGATAACAACCGAGTCCGGACTAATCTTG-3’
Reverse GA 5’-CAAGATTAGTCCGGACTCGGTTGTTATCCTCAGCG-3’

Mutant L510S

Direct LA 5’-GCTCGGAAAAAGAATGGATTCGATTGGCGTTCCAGAAGC-3’
Reverse LA 5’-TGCTTCTGGAACGCCAATCGAATCCATTCTTTTTCCGAGC-3’

Mutant W519R

Direct WA 5’-GATTTGATTGGCGTTCCAGAAGCACAACGGGGTAAAATAAGAGAAG-3’
Reverse WA 5’-CTTCTCTAATTTTACCCCGTTGTGCTTCTGGAACGCCAATCAAATC-3’

Mutant A550V

Direct AV 5’-CCCGCTTATTGCCGAAGTGAAAAAGCTTGTTGGAG-3’
Reverse AV 5’-CTCCAACAAGCTTTTTCACTTCGGCAATAAGCGGG-3’